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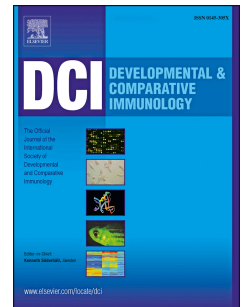
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**Induction of interferon and cell death in response to cytosolic DNA in chicken macrophages**

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**Abstract**

Responses to cytosolic DNA can protect against both infectious organisms and the mutagenic effect of DNA integration. Recognition of invading DNA is likely to be fundamental to eukaryotic cellular life, but has been described only in mammals. Introduction of DNA into chicken macrophages induced type I interferon mRNA via a pathway conserved with mammals, requiring the receptor cGAS and the signalling protein STING. A second pathway of cytosolic DNA recognition in mammalian macrophages, initiated by absent in melanoma 2 (AIM2), results in rapid inflammasome-mediated pyroptotic cell death. AIM2 is restricted to mammals. Nevertheless, chicken macrophages underwent lytic cell death within 15 min of DNA transfection. The mouse AIM2-mediated response requires double stranded DNA, but chicken cell death was maintained with denatured DNA. This appears to be a novel form of rapid necrotic cell death, which we propose is an ancient response rendered redundant in mammalian macrophages by the appearance of the AIM2 inflammasome. The retention of these cytosolic DNA responses through evolution, with both conserved and non-conserved mechanisms, suggests a fundamental importance in cellular defence.

Keywords: cytosolic DNA, chicken, interferon, STING, cell death, cGAS

**Abbreviations**

AIM2 absent in melanoma 2  
BMM bone marrow-derived macrophage  
cGAS cyclic GMP-AMP synthase  
CSF-1 macrophage colony stimulating factor-1  
CT DNA calf thymus DNA  
DMSO dimethyl sulfoxide  
dsDNA double stranded DNA  
dsRNA double stranded RNA  
GAPDH glyceraldehyde 3-phosphate dehydrogenase  
HI-FCS heat inactivated fetal calf serum  
IFI16 interferon inducible protein 16  
IFN interferon  
MDA5 melanoma differentiation-associated gene 5  
MLKL mixed-lineage kinase-like  
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
PAMP pathogen-associated molecular pattern  
PBS phosphate-buffered saline  
PI propidium iodide  
PYHIN pyrin and HIN domain-containing  
RIG-I retinoic-acid induced gene 1  
RIPK3 receptor interacting protein kinase 3  
siRNA small interfering RNA  
STING stimulator of interferon genes  
ZBP1 Z DNA binding protein 1

## 1. Introduction

Cells of the vertebrate innate immune system recognise pathogen-associated molecular patterns (PAMPs) to distinguish non-self from self. PAMPs alert immune cells to the presence of infection and initiate anti-infective responses including induction of type I interferons (IFNs) and inflammatory cytokines, and in some cases, cell death. Nucleic acids are important PAMPs, as they permit surveillance for viruses that can evolve rapidly to evade detection through other routes. Endosomal DNA and RNA can be recognised by toll-like receptors, on the basis of differential self and foreign base modifications (Stacey et al., 2003; Tluk et al., 2009). DNA and double stranded (ds) RNA are also recognised in the cytosol of cells (Ishii et al., 2006; Roberts et al., 2009; Yoneyama et al., 2004). Cytosolic DNA is detected in a sequence-independent manner on the basis of its aberrant location, and consequently both self and foreign DNAs elicit responses. Cytosolic DNA may be pathogen-derived, but could also result from gross nuclear or mitochondrial damage, or deregulated activity of endogenous retrotransposons.

In mouse and human cells cytosolic DNA recognition has at least two outcomes. Firstly, AIM2, a member of the PYHIN/HIN-200 family, recognises DNA in the cytosol and induces formation of an inflammasome complex, activating caspase-1 (Hornung et al., 2009; Roberts et al., 2009). Caspase-1 then cleaves precursors of inflammatory cytokines, as well as initiating a lytic cell death termed pyroptosis (Miao et al., 2011). The rapid osmotic lysis in pyroptosis distinguishes it from apoptosis, where membrane integrity is maintained until the dying cells are phagocytosed *in vivo*. A second pathway initiated by cytosolic DNA elicits the induction of type I IFNs (Ishii et al., 2006; Stetson and Medzhitov, 2006). Many different proteins have been suggested as receptors for DNA in this pathway, including Z DNA binding protein-1 (ZBP1/DAI), the helicase DDX41, and another member of the PYHIN/HIN-200 family, IFI16 (Cavlar et al., 2012; Unterholzner et al., 2010). It is now clear that STING, identified as a receptor for cyclic dinucleotide second messengers of bacteria (Burdette et al., 2011), is a signalling component in the response to DNA (Ishikawa et al., 2009) and cyclic GMP-AMP synthase (cGAS) is the essential cytoplasmic receptor (Sun et al., 2013). Upon recognition of dsDNA, cGAS catalyses synthesis of cyclic GMP-AMP that acts as a second messenger recognised by STING.

Genes with some homology to cGAS and STING can be found even in a choanoflagellate (Wu et al., 2014). However, only vertebrate cGAS homologues have the zinc thumb domain that is involved in binding DNA (Civril et al., 2013; Wu et al., 2014). In addition, IFN genes that are induced by cGAS recognition of DNA are only found in vertebrates (Pestka et al., 2004; Schultz et al., 2004). The inflammasome response to DNA mediated by AIM2 is operational only within certain mammals; non-mammals as well as bats have no PYHIN family proteins, and AIM2 has been independently lost in several mammalian lineages, generating pseudogenes in cow, llama, dolphin, dog, sheep, pig and elephant (Cridland et al., 2012). Thus if cytosolic DNA-induced cell death is an important defence, it must occur through different means in different species. Recently we demonstrated that cells of *Drosophila melanogaster* died in response to cytosolic DNA (Vitak et al., 2015), despite the absence of AIM2 and inflammasome machinery. Death was rapid and lytic and therefore non-apoptotic. Here we investigated the responses of chicken macrophages to cytosolic DNA. Birds lack all DNA-responsive PYHIN proteins including AIM2,

which is the only characterised DNA receptor initiating cell death (Cridland et al., 2012; Roberts et al., 2009). Hence any capacity for DNA-induced cell death in chickens would indicate the existence of an uncharacterised pathway. In addition, although chicken has a discernable cGAS orthologue, its role in induction of type I IFN has not been confirmed. We thus aimed to investigate both DNA-dependent lytic cell death and induction of IFN- $\beta$  mRNA in chicken macrophages.

## 2. Materials and methods

### 2.1 Cell culture

Chicken macrophage-like cell line HD-11 (Beug et al., 1979) was cultivated at 37°C, 5% CO<sub>2</sub> in complete RPMI-1640 (RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (HI-FCS), 1x GlutaMAX, 50 U/ml penicillin, 50 µg/ml streptomycin (all reagents from Life Technologies)). For maintenance and long incubation during knock-down experiments heat inactivated chicken serum (Life Technologies) was added to 1% final concentration. Bone marrow was obtained from the femurs of new-born male chicks, under approval from the University of Queensland animal ethics committee. Chicken bone marrow-derived macrophages (BMMs) were obtained by cultivation of marrow for one week in complete RPMI-1640 supplemented with 15% conditioned medium from CHO cells transiently expressing chicken CSF1 (Garceau et al., 2010), generated as follows. CHO suspension cells were grown in CD CHO medium (Gibco) supplemented with 8 mM glutamine (Gibco). The chicken CSF1 expression plasmid pEF-cCSF1 was transfected into CHO cells by pre-mixing 60 µg plasmid and 240 µg of linear polyethylenimine (PEI) in 5 ml of Opti-mem (Gibco) before adding to 30 ml of CHO cells grown to a density of  $1 \times 10^6$  cells per ml. After 4 h of incubation with the transfection reagent, cells were pelleted by centrifugation for 10 min at 160g and resuspended in 30 ml of CD-CHO containing 8 mM Glutamax, 100 U/ml penicillin, 100 µg/ml streptomycin, 7.5% CHO CD Efficient Feed A (Gibco), and 7.5% CHO CD Efficient Feed B (all reagents from Life Technologies). Cells were then incubated for 7 days at 37°C, 5% CO<sub>2</sub>, with shaking at ~120 rpm. After 7 days cells were removed by centrifugation for 10 min at 6,000g and the supernatant was filtered. Mouse BMMs were cultivated in complete RPMI-1640 with  $10^4$  U/ml CSF-1 as previously described (Sester et al., 2005), under approval from the University of Queensland animal ethics committee.

### 2.2 Nucleic acids

Calf thymus (CT) DNA, salmon sperm DNA and *E. coli* DNA were purchased from Sigma Aldrich and purified as described (Stacey et al., 2003). High molecular weight polyinosinic:polycytidylic acid (poly(I:C)) was obtained from Invivogen. DNA concentrations were either estimated by A260, or PicoGreen assay (Life Technologies) that specifically measures dsDNA.

### 2.3 Electroporation

Chicken BMMs and HD-11 cells were electroporated with various nucleic acids in 400 µl of growth medium at 260 V, 500 µF using a BioRad GenePulser MX. Mouse BMMs were electroporated at 240 V, 1000 µF. Cells were incubated at room temperature with nucleic acid for 10 min prior to electroporation, unless otherwise

stated.

#### 2.4 Assessment of IFN induction by real time PCR

Cells were electroporated at  $3.75 \times 10^7$  cells/ml, transferred into 15 ml of medium and split into four plates. Plates were incubated for 1, 2, 4 and 6 h followed by RNA extraction and cDNA synthesis with oligo(dT) priming as described (Roberts et al., 2009). Control reactions omitting reverse transcriptase were always performed to ensure lack of DNA contamination, since bird type I IFNs are single exon genes. Expression of IFN- $\alpha$ , - $\beta$ , STING, cGAS and GAPDH mRNAs in samples were determined by real time PCR using  $\Delta C_t$  analysis as described (Roberts et al., 2009). Primer sequences designed to the indicated transcripts were:

ggIFN- $\alpha$  (ENSGALT00000021627) CAACGACACCATCCTGGACA;  
GGGCTGCTGAGGATTTTGAA;  
ggIFN- $\beta$  (ENSGALT00000039477) TCCTGCAACCATCTTCGTCA;  
CACGTCTTGTTGTGGGCAAG;  
ggSTING (XM\_001232170) CACCTGGCGACTCTCTTTCTC;  
GAAGTTTGAGACAATCTTTCCTGC;  
ggcGAS (XM\_419881) CTACTGGTCCTTGCCCTTGG;  
GCCGTGAGCGACATTCTTCT;  
ggGAPDH (NM\_204305.1) CCTAGGATACACAGAGGACCAGGTT;  
ATTCAGTGCAATGCCAGCAC

#### 2.5 siRNA-mediated gene knock-down

Cells were harvested and resuspended at  $2.5 \times 10^7$  cells/ml. siRNA against target or control genes (15  $\mu$ l of 1M siRNA) were mixed with 400  $\mu$ l of cells, incubated at room temperature for 5 min and then electroporated as per section 2.3. Cells were then washed with RPMI medium and incubated at 37°C for 24 h. To test induction of type I IFNs cells were harvested and resuspended in 1.4 ml of RPMI, and 400  $\mu$ l was electroporated with 3  $\mu$ g of DNA or the same volume of phosphate-buffered saline (PBS) or left untreated. Cells were then plated in 2 ml of medium and incubated for 3.5 h at 37°C, followed by RNA extraction and cDNA synthesis. Sequences of Stealth RNAi siRNA (Life Technologies) were:

chicken STING#1 GGUCCUACUACAUCGGCUACCUGAA;  
chicken STING#2 UGUUAUGUGAUCAGAGAUAAAGGACAA;  
chicken cGAS#1 GGAGAGAGUUCAGAUUAAACCAAU;  
chicken cGAS#2 GAUUCCAGUAUUUACCCAGGUGUA;  
mouse Casp9 UGGGACCAAUGGGACUCACAGCAAA

An siRNA targeting mouse Caspase 9 with no significant chicken homology was used as a control.

#### 2.6 MTT assay for viability

Cells were electroporated at  $2.5 \times 10^6$  cells/ml and 100,000 cells were then plated in quadruplicate in 96-well tissue culture plates for viability measurement. Reduction of the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble blue product was used as an indicator of cell viability (Berridge and Tan, 1993). MTT was added to a final concentration 1 mg/ml, and cells were incubated for 1 h at 37°C. After incubation, cells were solubilised overnight by adding an equal volume of 10% Triton X-100 and 0.1N HCl in isopropanol. Absorbance was measured at 570 nm. To compensate for any cell number differences between experiments, MTT assay data is presented normalised to the control sample.



## 2.7 Flow cytometric analysis of cell death

Cells were electroporated at  $1 \times 10^6$  cells in 375  $\mu$ l of complete RPMI-1640 with 25 mM HEPES and 25  $\mu$ l of PBS containing nucleic acid. Cells (300  $\mu$ l) excluding floating cell debris generated by the electrical pulse were rapidly transferred to 1.5 ml microfuge tubes containing 950  $\mu$ l of complete RPMI-1640 with 25 mM HEPES. For DNA dose response, cells were incubated for 30 min at 37°C then a final concentration of 1  $\mu$ g/ml propidium iodide (PI) was added prior to analysis by flow cytometry on a BD Accuri C6. For real-time flow cytometry (Stacey et al., 2016), the 950  $\mu$ l of complete RPMI-1640 with 25 mM HEPES at 37°C or 4°C as indicated, was supplemented with 1.5  $\mu$ g PI prior to addition of cells. Cells were maintained at 37°C on a heating block or in an ice/water bath, as indicated, during analysis on a BD Accuri C6 flow cytometer.

### 3. Results

#### 3.1 Cytosolic DNA induces IFN- $\beta$ mRNA in chicken macrophages.

To determine whether introduction of cytosolic DNA induces chicken type I IFN expression, chicken HD-11 macrophage-like cells were electroporated with calf thymus (CT) DNA and also the synthetic dsRNA, poly(I:C), a known inducer of chicken IFN- $\beta$  via cytosolic recognition (Karpala et al., 2011) (Fig. 1A). An initial experiment used 20  $\mu$ g of DNA or poly(I:C), but after finding that the DNA compromised viability, subsequent experiments used 5  $\mu$ g. IFN- $\beta$  mRNA was clearly induced by both DNA and poly(I:C), with the DNA response peaking at 4 h post electroporation. Poly(I:C) gave a low induction of IFN- $\alpha$ , whereas the DNA response was minimal or absent (Fig. 1A). The primers for IFN- $\alpha$  were designed to the currently annotated IFNA3 transcript (NM\_205427.1), but will recognise other predicted IFNA transcripts. However, the IFNA loci in chicken are poorly annotated, and it is possible that some genes are not amplified well with these primers. Similar experiments were performed using primary chicken BMMs from 4 individual birds (Fig. 1B). Results were largely comparable to the cell line, although poly(I:C) dramatically induced the level of IFN- $\alpha$  in the primary cells. There was some inter-individual variability, consistent with the known variation in induction of these genes, which are located on the Z chromosome, and exhibit some sex-specific regulation (Garcia-Morales et al., 2015; Nanda et al., 1998). Overall the induction of type I IFN in chicken macrophages by transfected dsRNA and DNA resembles that seen in mammals, although the level of induction achieved with DNA was relatively low.

#### 3.2 cGAS and STING are required for chicken response to DNA

Given the importance of cGAS and STING in mammalian recognition of cytoplasmic DNA (Ishikawa et al., 2009; Sun et al., 2013) and antiviral responses (Schoggins et al., 2014) we investigated the roles of the chicken orthologues. STING and cGAS were knocked down in HD-11 cells and the subsequent response to electroporated DNA analysed. The level of induced IFN- $\beta$  expression, as well as STING and cGAS knock down efficiencies were measured by real time PCR (Fig. 2). A consistent knock down of 50-60% of cGAS mRNA (Fig. 2C) was sufficient to reduce induction of IFN- $\beta$  after electroporation with DNA by 80-90% (Fig. 2A). Inhibition of IFN- $\beta$  induction was also observed with STING knockdown, and correlated with the efficiency of the two siRNAs (Figs 2A and B). A control siRNA had no effect on IFN- $\beta$  induction. These data support the hypothesis that the identified homologues of cGAS and STING play the same role in birds and mammals.

#### 3.3 Rapid chicken cell death induced by cytosolic DNA.

The second characterised mammalian response to cytosolic DNA is activation of the AIM2 inflammasome, leading to cell death and IL-1 $\beta$  production. However, our previous study showed that electroporated DNA also induced rapid cell death in *Drosophila* cells, where AIM2 is lacking (Vitak et al., 2015). To determine whether birds also have an alternative pathway of DNA-dependent cell death, we initially electroporated HD-11 cells with 10  $\mu$ g of CT DNA and assessed viability by MTT assay for mitochondrial activity. Even 1 h after electroporation, there was more than 50% reduction in MTT cleavage (Fig. 3A). To exclude possible contaminants as a

source of the toxicity, we treated CT DNA with DNase I, which abolished the cell death observed with CT DNA (Fig. 3B). The reduction in viability measured by MTT cleavage was dose-dependent, with toxicity increasing up to 20 µg of transfected DNA (Fig. 3C).

### 3.4 DNA and not dsRNA induces rapid cell death

To determine whether rapid cell death was specific to transfected DNA, or was also seen with other polyanions we examined the effect of electroporated poly(I:C) (dsRNA). In mammalian cells, transfected synthetic dsRNA is a potent inducer of apoptotic cell death (Jordanov et al., 2005). The apoptotic response of mouse macrophages to transfected dsRNA occurs more slowly than the rapid pyroptosis induced by DNA; a reduction in MTT cleavage was seen at 3 h but not 1 h post dsRNA transfection (Vitak et al., 2015). HD-11 cells showed toxicity of DNA and not dsRNA at both 1 h and 3 h post transfection, confirming the specific nature of the response to DNA (Fig. 3D). The lack of effect of dsRNA after 3 h contrasts with results obtained with mouse BMMs (Vitak et al., 2015). The effects of DNA were not dependent upon its origin; DNAs from a mammal (calf thymus), fish (salmon sperm) and a prokaryote (*E. coli*) were similarly toxic (Fig. 3E).

### 3.5 Denatured DNA retains toxicity for chicken cells

The mammalian AIM2 response depends on double stranded DNA; either synthetic single stranded DNA, or genomic DNA denatured by boiling was unable to initiate cell death in mouse macrophages (Roberts et al., 2009; Stacey et al., 1993). To examine the effect of DNA structure on HD-11 cell death, we boiled DNA for 10 min, transferred it onto ice and performed transfection within 2 min. Under these conditions mouse macrophages were killed by intact DNA, but not denatured DNA, as expected (Fig. 3F). By contrast, the same preparations of denatured DNA were able to kill HD-11 cells as effectively as intact DNA (Fig. 3F). In this respect, the response of chicken cells resembles that of *Drosophila* (Vitak et al., 2015).

### 3.6 DNA, but not dsRNA is acutely toxic for chicken BMMs

Transfection of chicken BMMs was performed to confirm that primary cells respond similarly to the immortalised HD-11 cell line. Intact or DNase I-digested CT DNA, or poly(I:C) were electroporated into chicken BMMs and cell viability was assessed at 1 h and 3 h following transfection, based upon the metabolic activity detected by cleavage of MTT (Fig. 3G). Primary macrophages were killed by cytosolic DNA by 1 h post transfection. Similar to the HD-11 cell line, chicken BMMs did not die in response to transfected poly(I:C) 1 h post electroporation, however unlike HD-11 a low level of toxicity was observed 3 h post transfection (Fig. 3D).

### 3.6 Transfected DNA induces non-apoptotic lytic cell death

The pyroptotic response elicited by AIM2 in mouse macrophages can be detected within 1 h after DNA electroporation, by staining with propidium iodide (PI), a membrane-impermeable DNA stain (Sagulenko et al., 2013; Yin et al., 2013). In the absence of caspase-1 which elicits pyroptosis, AIM2 triggers apoptosis, but membrane integrity is still maintained in the apoptotic cells at 1 h post electroporation

(Sagulenko et al., 2013). The loss of membrane integrity was evident in both mouse macrophages and HD-11 within 30 min of DNA electroporation (Fig. 4A) but occurred at lower DNA concentration in the chicken cells. We further investigated the kinetics of membrane permeability to PI following electroporation of DNA by real-time flow cytometry (Stacey et al., 2016). Electroporation alone or addition of DNA to cells without electroporation did not increase permeability to PI. The addition of DNA slightly increased the staining of all viable cells, presumably due to cell-surface DNA binding, and decreased the intensity of signal from dead cells due to sequestration of PI by free CT DNA in the culture medium (Fig. 4B). Electroporation with DNA caused a profound increase in PI positive cells, with loss of membrane integrity detected between 3 and 15 min after electroporation of HD-11 cells, chicken BMMs or mouse BMMs. The rapid cell death did not occur in murine BMMs derived from *Casp1*<sup>-/-</sup> mice (Fig. 4B). When cells were cooled and maintained at 4°C immediately following electroporation with DNA, membrane integrity of both murine BMMs and HD-11 cells was maintained for up to 20 min (Fig. 4B). However if these cells were subsequently warmed to 37°C, they became rapidly permeable to PI. Together these data suggest the rapid cell death observed in chicken macrophages is an active process, similar to AIM2-dependent pyroptosis, and not an artifact resulting from direct physical damage to cells by transfected long DNA.

#### 4. Discussion.

We have demonstrated two pathways of response to cytosolic DNA in chicken cells – induction of type I IFN, and cell death. DNA-dependent IFN induction appears to have been present in the common ancestor of birds and mammals. cGAS and STING, key proteins of the mammalian IFN induction pathway were identified throughout vertebrates (Wu et al., 2014). Knockdown of STING or cGAS in chicken HD-11 cells confirmed the requirement for this pathway in induction of type I IFN mRNA in response to cytosolic DNA. Bird cGAS molecules lack an unstructured N-terminal domain, and STING has a shorter N-terminal transmembrane domain than its mammalian counterparts (Wu et al., 2014), but the avian molecules are clearly still functional in IFN induction. Mammalian cGAS is required for induction of IFN in response to viruses such as modified vaccinia virus Ankara (Dai et al., 2014), adenovirus (Lam and Falck-Pedersen, 2014), HSV-1 (Li et al., 2013), and HIV (Lahaye et al., 2013). The cGAS DNA recognition pathway may therefore be important in the response to a range of chicken DNA viruses such as fowlpox and Marek's disease virus as well as retroviruses which generate cDNA within the cytoplasm.

Whilst it was possible to confirm the function of cGAS in the DNA response in chicken, the level of IFN- $\beta$  mRNA induced was low. We note that STING mRNA was barely detectable, and it is possible that use of other cell types, or priming stimuli for prior induction of STING may be necessary to observe a strong response to cytosolic DNA. Additionally, chicken cGAS may recognise specific sequences or structures enriched in pathogenic DNA and scarce in CT DNA. Although human cGAS can bind to any long dsDNA sequence, it apparently also binds specifically to a short stretch of HIV DNA with the presence of unpaired terminal guanidines (Herzner et al., 2015). If such specific requirements exist for chicken cGAS, they remain to be identified.

Previous studies on IFN induction in chicken, have focused on RNA viruses, including influenza A and Newcastle disease virus (Staeheli et al., 2001). The mammalian receptors for dsRNA are retinoic-acid induced gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), which recognise short and long dsRNA, respectively (Kohlway et al., 2013). Although both genes are present in duck, chicken has only MDA5, which led to speculation that lack of RIG-I results in differential susceptibility to influenza virus between chickens and ducks (Barber et al., 2010; Karpala et al., 2011). However, chicken MDA5 appears to recognise both short and long dsRNA (Hayashi et al., 2014; Karpala et al., 2011) and may at least partially substitute for the function of RIG-I. We found that the induction of type I IFN in both HD-11 cells and chicken macrophages was much higher with dsRNA than DNA. Mammalian cells also show a greater sensitivity to electroporated dsRNA than DNA for induction of IFN- $\beta$ . Long regions of dsRNA are intrinsically foreign, and a sensitive detection system may operate without risk. However, DNA may escape from phagosomes or other sites in normally functioning cells, and a higher threshold for detection of DNA may be needed to avoid unwarranted responses. Indeed, the impact of the loss of the cytosolic DNase, Trex1, revealed that basal degradation of cytosolic DNA is required to prevent IFN-dependent inflammation and autoimmunity (Stetson et al., 2008).

Type I IFNs are found throughout vertebrates, and phylogenetic analysis suggests that the last common ancestor of birds, mammals and fishes had a single gene ancestral to all the type I IFNs (Pestka et al., 2004; Schultz et al., 2004). These gene families thus appear to have amplified and diverged independently within these lineages. Consequently the chicken genes for IFN- $\alpha$  and - $\beta$  are not thought to be directly orthologous to the mammalian genes of the same names, and in some publications are called IFN1 and 2 to distinguish them from the mammalian genes. However, like mammals, there is a single chicken IFN- $\beta$  gene and a family of IFN- $\alpha$  genes (Goossens et al., 2013; Sick et al., 1996) and these are thought to differ in their regulation (Schultz et al., 2004). Indeed here we saw discordant regulation, since IFN- $\alpha$  mRNA was poorly induced in HD-11 and well induced in primary macrophages by poly(I:C), although IFN- $\beta$  mRNA was comparably induced in both cell types. The difference between primary cells and the cell line may stem from the immortalisation of HD-11 with avian myelocytomatosis virus MC29 (Beug et al., 1979), but could also result from genetic variation in promoter sequences; the presence of these genes on the Z chromosome, and their haploid state in the female may allow rapid evolutionary selection (Garcia-Morales et al., 2015; Nanda et al., 1998).

Cell death is a fundamental defence against infection with intracellular pathogens - it can prevent replication, and release organisms from a protective niche. In mouse macrophages, detection of pathogen DNA by AIM2 can elicit lytic pyroptotic death. Despite lack of AIM2 in chicken cells (Cridland et al., 2012), transfection of DNA resulted in lytic death on a similar time course to mouse macrophages. This was not a non-specific response to a polyanion, as dsRNA did not induce death at 1 h after electroporation. A key difference between the mouse and chicken macrophage responses is the retention of chicken cell death with denatured DNA. Whether this reflects sensitivity to single stranded DNA, or a very high sensitivity to rapidly reannealing repeat sequences remains to be determined. This feature was also seen in DNA-induced death of *Drosophila* cells (Vitak et al., 2015), and suggests a very



distinct mode of DNA recognition to that mediated by AIM2, which is strictly dependent on dsDNA. The similarity of the chicken and *Drosophila* responses suggests the existence of an evolutionarily ancient mechanism of defence against stray DNA, that has been superseded by AIM2 in macrophages from some mammalian species. Indeed, *Aim2*<sup>-/-</sup> mouse macrophages show no cell death under conditions used here (see supplementary data in (Yin et al., 2013)), and thus have lost this alternative pathway.

The observed death of chicken cells in response to DNA is rapid and lytic. These characteristics are observed in mammalian pyroptosis, and programmed necrosis (necroptosis). DNA-induced pyroptosis involves assembly of an AIM2 inflammasome with recruitment of the adapter molecule ASC and procaspase-1. Whilst it is conceivable that a DNA receptor other than AIM2 could elicit an inflammasome response and caspase-1-mediated cell death in chicken, inflammasomes are yet to be characterised in birds (Chen et al., 2013). The central death domain-containing inflammasome adapter molecule, ASC, which recruits caspase-1, has no discernible orthologue in bird genomes, whereas ASC homologues can be found in reptiles, amphibians and fishes. A caspase-1 orthologue is present in chicken (Johnson et al., 1998), and is expressed in immune tissues, such as bursa of Fabricius, spleen and bone marrow. It is not known to participate in death pathways, and pathways of activation remain to be established. In mammalian necroptotic pathways, phosphorylation of mixed-lineage kinase-like (MLKL) by receptor interacting protein kinase 3 (RIPK3), leads to exposure of the MLKL N-terminal domain and its membrane association, triggering rapid lytic death (Hildebrand et al., 2014). A homologue of *MLKL* is present in the chicken genome (Hildebrand et al., 2014). However, in *Drosophila melanogaster*, we cannot identify an *MLKL* homologue encoding the N-terminal 5 helix bundle that mediates cell death. Since *Drosophila* and chicken DNA-dependent cell death are morphologically similar and are both initiated by denatured DNA (Vitak et al., 2015), they are likely to be initiated through a common ancient mechanism. The lack of MLKL or direct caspase-1 orthologue in *Drosophila* suggests this is a novel pathway, not described in mammals.

In summary, the normal localisation of DNA in the nucleus and mitochondria allows detection of cytosolic DNA as an alarm system to indicate infection or cell damage. Chicken macrophages respond to cytosolic DNA with induction of type I IFN via the cGAS pathway conserved with mammals, and with lytic cell death by a novel pathway. Such responses have obvious relevance to infections, not only with DNA viruses, but also with retroviruses and cytosolic bacteria (Gao et al., 2013; Rathinam et al., 2010; Storek et al., 2015). At a low level of infection, induction of IFN is likely to block viral replication and alert neighboring cells. At higher levels of cytosolic DNA, a more extreme response such as rapid lytic cell death will curtail viral replication and promote inflammation. Apart from infection, cytosolic DNA may indicate gross cellular damage, or mutagenic (retro)transposon DNA. In both these cases, cell death may provide an appropriate defence. The fact that certain phagocytic immune cells of mammalian, chicken and *Drosophila* origin undergo DNA-dependent death by several different mechanisms, emphasises the likely importance of this process in maintaining organism integrity.

## Acknowledgements

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## Figure Legends

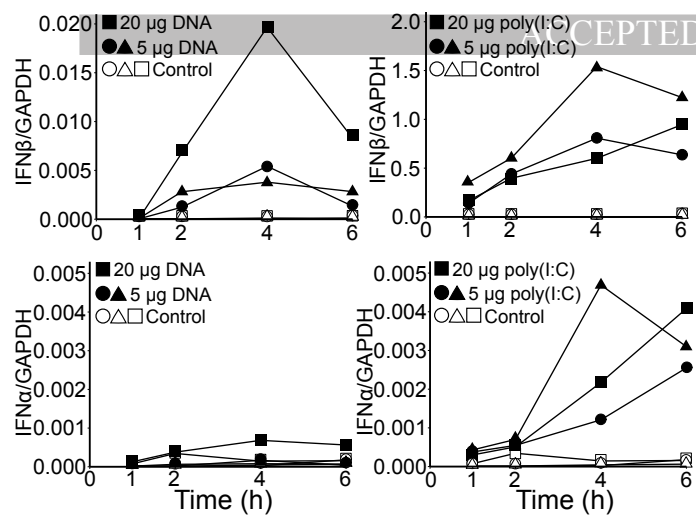
**Figure 1. Induction of type I IFNs in HD-11 and chicken BMMs after electroporation with nucleic acids.** mRNA levels were measured by real time PCR and are expressed relative to GAPDH mRNA. **A.** Induction of IFN- $\alpha$  and IFN- $\beta$  mRNA in HD-11 cell line after electroporation either without (control), or with the indicated amounts of poly(I:C) or CT DNA. Symbols show three separate experiments. **B.** Induction of IFN- $\alpha$  and IFN- $\beta$  mRNA in chicken BMMs after transfection without (control) or with 5  $\mu$ g of poly(I:C) or CT DNA. Symbols show four separate experiments using cells from different animals.

**Figure 2. STING and cGAS are involved in DNA-dependent IFN- $\beta$  induction in chicken.** **A.** Level of IFN- $\beta$  mRNA at 3.5 h after electroporation with CT DNA, in HD-11 cells that had been electroporated with or without the indicated siRNAs 24 h earlier. Results were normalised to the no siRNA +DNA sample for each experiment, to account for variable IFN- $\beta$  induction between experiments. Bars represent the mean  $\pm$  SEM of relative expression. Significance relative to the control siRNA +DNA sample was determined prior to normalisation by paired ratio one-tailed t test (N=3), \*  $p < 0.03$ . **B** and **C.** Knockdown of STING and cGAS mRNAs (respectively) at 24 h after electroporation with the indicated siRNAs. Results were normalised to the no siRNA samples, which had mean raw values of 0.000047 for STING/GAPDH and 0.0042 for cGAS/GAPDH. Bars represent the mean  $\pm$  SEM of relative expression. Significance relative to the no siRNA sample was determined prior to normalisation, by paired ratio one-tailed t test (N=3), \*  $p < 0.02$ .

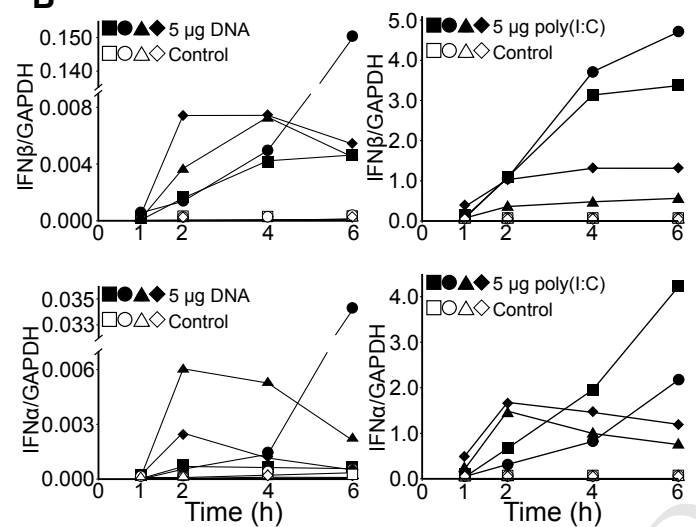
**Figure 3. Viability of chicken cells in response to DNA and dsRNA.** Viability of cells was measured by MTT cleavage at 1 h post electroporation unless indicated otherwise. **A.** DNA-induced cell death in HD-11 cells after electroporation with 10  $\mu$ g CT DNA. Bars represent data of 14 experiments (mean  $\pm$  SEM), normalised to the no DNA sample in all experiments. Statistical significance was determined prior to normalisation by paired one tailed t-test. \*\*\*\* $p < 10^{-11}$ . **B.** DNA-specific response of HD-11 cells. Cells were electroporated with 10  $\mu$ g CT DNA that was untreated or treated with DNase I. Bars represent data from 3 experiments (mean  $\pm$  SEM), normalised to the no DNA sample for each experiment. **C.** Dose-dependent response of HD-11 to electroporation with indicated amounts of DNA. Bars represent data from 3 experiments (mean  $\pm$  SEM), normalised to the no DNA sample for each experiment. **D.** HD-11 death in response to DNA, but not to synthetic dsRNA poly(I:C). HD-11 cells were electroporated with 10  $\mu$ g CT DNA or poly(I:C), and cleavage of MTT measured after 1 and 3 h. Bars represent data from 3 experiments (mean  $\pm$  SEM), normalised to the no DNA samples. **E.** Cytotoxic effect of DNA from different sources. 10  $\mu$ g of CT DNA, salmon sperm DNA or *E.coli* genomic DNA were used in electroporation. Bars represent results of two electroporations (mean  $\pm$  range). **F.** Denatured CT DNA induces cell death in HD-11 cells, but not mouse macrophages. CT DNA was left intact or boiled for 10 min, transferred to ice and 10  $\mu$ g used immediately for electroporation. Bars represent data from 3 (HD-11) or 2 (BMMs) experiments (mean  $\pm$  SEM, or mean  $\pm$  range, respectively), normalised to the no DNA sample. **G.** Response of chicken BMMs to DNA and dsRNA. Intact or DNaseI-digested CT DNA or poly(I:C) (all 10  $\mu$ g) were transfected via electroporation and viability was measured at 1 and 3 h after treatment using MTT cleavage. Bars represent data from 2 experiments (mean  $\pm$  range).

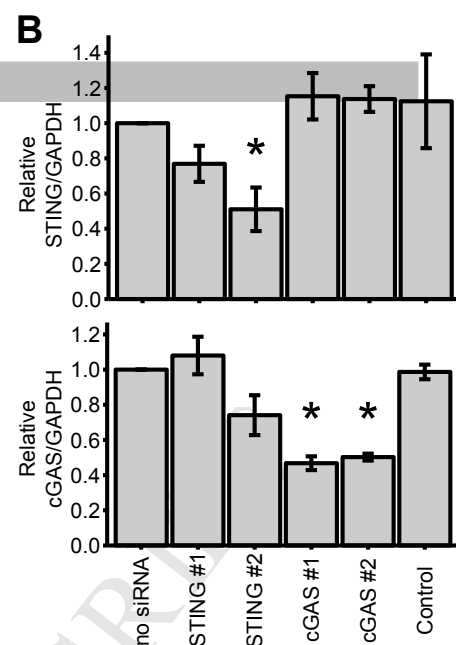
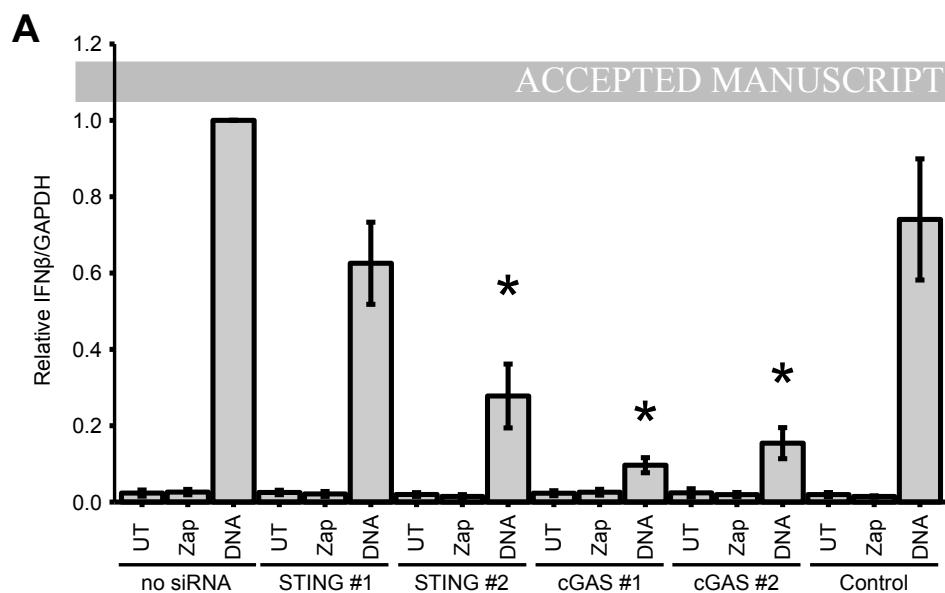
**Figure 4. The rapid lytic death of chicken macrophages is similar to mouse BMM pyroptosis.** **A.** DNA dose response for cell death of HD-11 cells and C57BL/6 BMMs following electroporation of CT DNA. HD-11 cells and mouse BMMs were electroporated with varying amounts of CT DNA, incubated at 37°C for 30 min and then analysed for cell viability by flow cytometry with PI. Data represent the mean  $\pm$  SEM. For HD-11 N=5-6, and for BMMs N=4 independent experiments. **B.** Rapid lytic cell death induced by DNA in chicken and mouse cells. HD-11 cells, mouse WT BMMs, caspase-1-deficient (*Casp1*<sup>-/-</sup>) BMMs and chicken BMMs were analysed by real-time flow cytometry over the indicated time period following either: no treatment (UT); electroporation alone (Zap); incubation with DNA for 10 min (DNA); or incubation with DNA for 10 min followed by electroporation (DNA + Zap). The two last columns show the temperature-dependent behavior of the DNA-induced cell death in HD-11 and mouse BMMs. For the “DNA+Zap 4°C” sample, cells were incubated with DNA for 10 min followed by electroporation and immediate transfer to an ice/water bath during analysis. For the “DNA+Zap 37°C after 4°C” sample, cells were treated as per the “DNA+Zap 4°C” sample for 25 min, and then transferred to 37°C during analysis. Amounts of DNA were 10  $\mu$ g for chicken cells and 20  $\mu$ g for mouse cells. Data is representative of 3 independent experiments for HD-11 and mouse WT BMMs and 2 experiments for *Casp1*<sup>-/-</sup> BMMs and chicken BMMs.

**A**

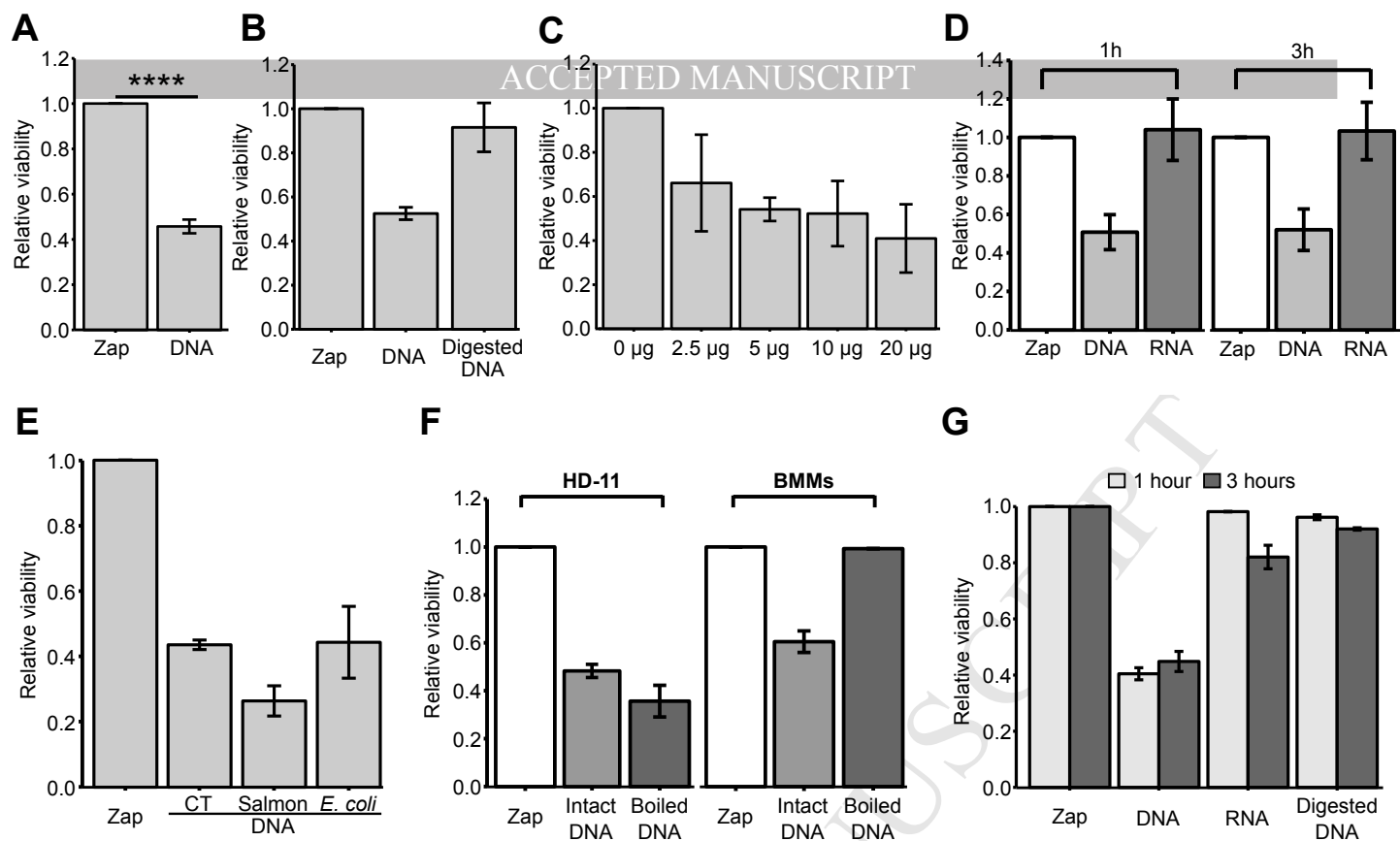


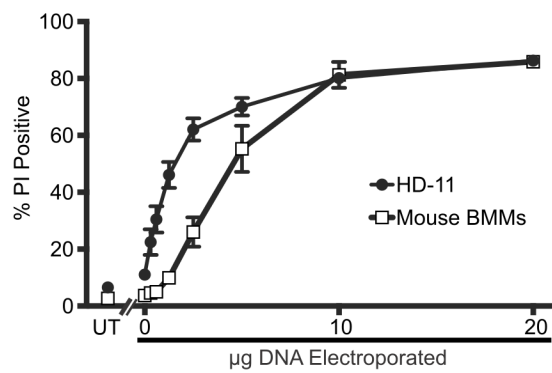
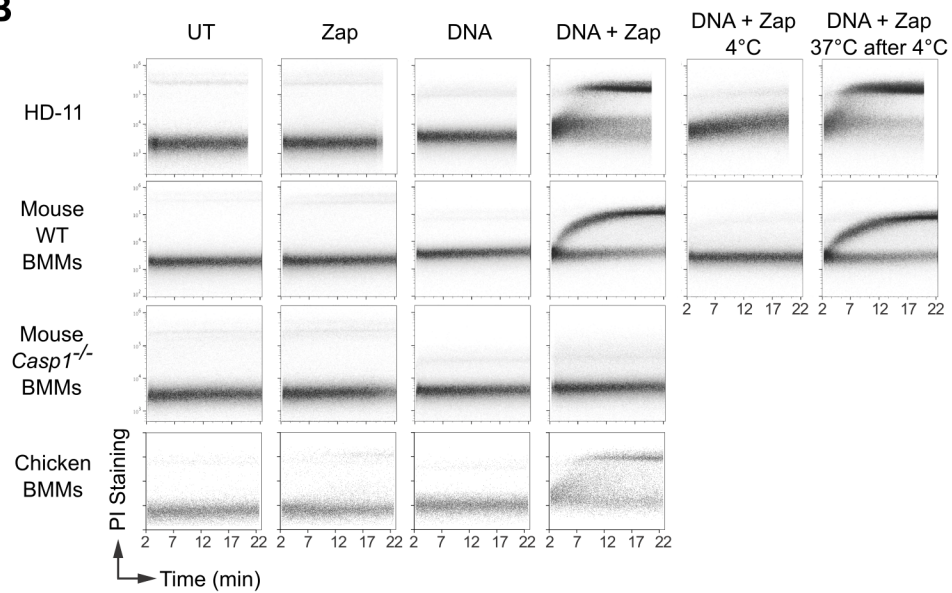
**B**









**A****B**

**Highlights**

- Cytosolic DNA induces cell death and interferon mRNA in chicken macrophages
- Induction of interferon-beta mRNA depends on cGAS and STING
- Cell death is rapid and lytic, not apoptotic
- Chickens lack AIM2, the mammalian initiator of DNA-dependent death
- Several mechanisms of DNA-dependent cell death have evolved independently